REMARKS

Reconsideration and withdrawal of the rejections of this application and consideration and entry of this paper are respectfully requested in view of the herein remarks and accompanying information, which place the application in condition for allowance.

I. STATUS OF CLAIMS AND FORMAL MATTERS

Claims 1-3, 5, 7, 11, 13, 15, 17, 19-41, 101, and 104-107 are currently under consideration. Claims 1, 2, 3, 28, 30-32, 34, 35, 37, 39-41, and 101 are amended, and claims 21, 24, 27, 33, and 42-100 are canceled without prejudice, without admission, without surrender of subject matter, and without any intention of creating any estoppel as to equivalents.

No new matter is added.

It is submitted that the claims herewith are patentably distinct over the prior art, and these claims are in full compliance with the requirements of 35 U.S.C. §112. The amendments to the claims presented herein are not made for purposes of patentability within the meaning of 35 U.S.C. §§§§ 101, 102, 103 or 112. Rather, these amendments and additions are made simply to clarify the scope of protection to which Applicant is entitled.

Amended claims 1, 2, 3, 40, 41, and 101 clarify the disclosed subject matter and are supported throughout the specification as originally filed. For instance, support for these claims can be found, as an example, on page 4, lines 2 and 13-19; page 27, line 8 - page 28, line 21; page 33, line 1-14; page 54, line 8-15, and page 96, lines 14-24. Claims 28, 30-32, 34, 35, 37, and 39 are amended to ensure proper antecedent basis or to correct references to other claims. Claims 42-100 were previously withdrawn and are herein canceled.

II. THE OBJECTION TO THE CLAIMS IS OVERCOME

Claim 39 remains objected for being dependent in part on canceled claim 4. By this paper, claim 39 is amended to eliminate the reference to claim 4.

Accordingly, reconsideration and withdrawal of the objection to the claims are respectfully requested.

III. REJECTION UNDER 35 U.S.C. §112, 1ST PARAGRAPH IS OVERCOME

Claims 1-3, 5, 7, 11, 13, 15, 17, 19-41, 101, and 104-107 were rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the enablement requirement. The rejection is respectfully traversed.

The Office Action asserts that the specification does not "reasonably provide enablement for an agent which operably engages the MHC which is any other substance, or a starting cell population which is of non-haematopoietic origin." According to the Office Action, the claims encompass agents which are broader in scope than antibodies which bind to the MHC receptor, and the specification allegedly provides no evidence that an agent which engages a receptor other than a MHC receptor could induce the enrichment of undifferentiated cells in a population comprising committed cells. The Office Action also alleges that the specification provides "no objective evidence that a cell population comprising committed cells from any other population other than haematopoietic cells can be used to provide an altered cell population comprising an increased number of undifferentiated cells." The Office Action cites references Almedida-Porada et al. (Rev Clin Exp Hematol 5:26-41, 2001) and Horwitz (Curr Opin Pediatrics 15: 32-37, 2003) which allegedly indicate that the art regarding stem cell plasticity is unreliable.

Applicant respectfully notes that, according to the Court of Appeals for the Federal Circuit in the case of *In re Wands*, 8 USPQ2d 1400 (Fed. Cir. 1988),

> Enablement is not precluded by the necessity for some experimentation such as routine screening. However, experimentation needed to practice the invention must not be undue experimentation. 'The key word is undue, not experimentation.' The determination of what constitutes undue experimentation in a given case requires the application of standard of reasonableness, having due regard for the nature of the invention and the state of the art. The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed ... [Citations omitted]. *Id.* at 1404.

Determining whether undue experimentation is required to practice a claimed invention turns on weighing many factors summarized in *In re Wands*, 858 F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988), for example: (1) the quantity of experimentation necessary; (2) the amount of direction or guidance presented; (3) the presence or absence of working examples of the invention; (4) the nature of the invention; (5) the state of the prior art; (6) the relative skill of

those in the art; (7) the predictability or unpredictability of the art; and (8) the breadth of the claims.

Thus, it is respectfully submitted that for a proper Section 112, first paragraph, lack of enablement analysis, an Office Action must show that the *Wands* factors are not met. Simply, it is respectfully asserted that the lack of enablement rejection fails to provide a fact based analysis using the *Wands* factors that supports the proposition the claimed invention require **undue** experimentation.

The Examiner is respectfully reminded that a specification need not contain any example of the invention, as the issue is whether the disclosure enables one skilled in the art to practice the invention without undue experimentation. *In re Borkowski*, 422 F.2d 904, 164 USPQ 642 (CCPA 1970). Simply, a determination that undue experimentation is necessary to practice the invention does not necessarily follow from a lack of examples in the specification. And, the Examiner is further respectfully reminded that an applicant need not describe all actual embodiments of a claimed invention.

Applicant initially draws attention to the claims amended herein, notably claims 1, 2, 3, 40, 41, and 101 which are clarified to recite that the cell population comprises haematopoietic cells, and that the agent is selected from the group consisting of (a) an antibody that binds to MHC antigens, (b) erythropoietin, and (c) GM-CSF. In view of these amendments, Applicant respectfully asserts that the instant claims are enabled by the specification.

Firstly, the breadth of the claims is herein revised since the amendments clarify the subject matter related to the cell population and the agent used with the claimed device. As such, in contrast to the Office Action's allegations, the claims herein do not refer to a staring cell population "which is of non-hematopoietic origin" or an agent "which is any other substance." Hence, considering the revised scope of the claims, Applicant asserts that the instant claims do not lack enablement.

The specification also provides substantial guidance and working examples for performing the claimed invention. For example, uses of the present invention involving haematopoietic cell population are discussed on page 48, beginning at line 15, and working examples for using a haematopoietic cell population are presented in protocols beginning on page 54, line 8. Further, protocols for using antibodies of MHC are shown in the working examples on page 56, beginning at line 19, and on page 57, beginning at line 24. Examples

related to the use of erythropoietin and GM-CSF are provided on page 96, beginning at line 14. Considering that these citations from the specification demonstrate how the claimed invention can be used, it is clear that the specification sufficiently provide guidance and working examples for using the present invention.

In addition, a skilled artisan, in view of the state of the art, would be capable of using the device of the claimed invention without undue experimentation. Abuljadayel (Curr Med Res Opin 19: 355-75, 2003) describes using CR3/43 monoclonal antibody (antibody that binds to MHC antigens) to convert mononuclear cells from a blood donor (haematopoietic cell population) to pluripotent stem cells (thereby increasing the relative number of undifferentiated cells). Thus, the state of the art clearly indicates that a device using the cell population and agents as instantly claimed can be practiced by one skilled in the art without undue experimentation.

Finally, Applicant notes that the Office Action recites "the specification, while being enabling for device comprising a means for introducing into a chamber an agent which is an anti-MHC antibody which operabl[y] engages the MHC receptors in a population of committed cells which are hematopoietic cells and results in an increase in the relative number of hematopoietic cells..." Consequently, the Office Action admits that a cell population comprising haematopoietic cells as instantly claimed in the present invention is enabled by the specification.

Therefore, in view of the breadth and scope of the instant claims, the amount of guidance provided, the working examples, and the state of the art, as well as statements by the Office Action, the instant claims fulfill the enablement requirement. Accordingly, reconsideration and withdrawal of the rejection of claims under 35 U.S.C. 112, first paragraph are respectfully requested.

REQUEST FOR INTERVIEW

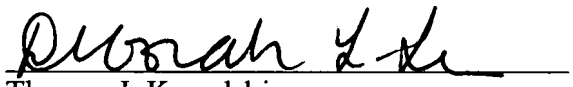
If any issue remains as an impediment to allowance, an interview with the Examiner and SPE are respectfully requested and the Examiner is additionally requested to contact the undersigned to arrange a mutually convenient time and manner for such an interview.

CONCLUSION

In view of the remarks and amendments herewith, the application is in condition for allowance. Favorable reconsideration of the application and prompt issuance of a Notice of Allowance are earnestly solicited. The undersigned looks forward to hearing favorably from the Examiner at an early date, and, the Examiner is invited to telephonically contact the undersigned to advance prosecution.

Respectfully submitted,

FROMMER LAWRENCE & HAUG LLP

By: 

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ORIGINAL ARTICLE

Induction of stem cell-like plasticity in mononuclear cells derived from unmobilised adult human peripheral blood

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Key words: Haematopoietic, cardiomyocyte, neuronal progenitor/stem cells – CR3/43 monoclonal antibody – Embryoid body – Mononuclear cells – Pluripotency, transdifferentiation – Retrodifferentiation – Undifferentiated/embryonic stem cells

SUMMARY

Undifferentiated pluripotent stem cells with flexible developmental potentials are not normally found in peripheral blood. However, such cells have recently been reported to reside in the bone marrow. Herein are reported methods of inducing pluripotency in cells derived from unmobilised adult human peripheral blood. In response to the inclusion of purified CR3/43 monoclonal antibody (mAb) to well-established culture conditions, mononuclear cells (MNC) obtained from a single blood donor are converted into pluripotent haematopoietic, neuronal and cardiomyogenic progenitor stem cells or undifferentiated stem cells. The haematopoietic stem cells are CD34+, clonogenic and have been shown to repopulate non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. The neuronal precursors transcribe the primitive stem cell markers OCT-4 and nestin, and on maturation, differentially stain positive for neuronal, glial or oligodendrocyte-specific antigens. The cardiomyogenic progenitor stem cells form large bodies of asynchronously beating cells and differentiate into mature cardiomyocytes which transcribe GATA-4. The undifferentiated stem cells do not express haematopoietic-associated markers, are negative for major histocompatibility complex (MHC) class I and II antigens, transcribe high levels of OCT-4 and form embryoid body (EB)-like structures. This induction of stem cell-like plasticity in MNC may have proceeded by a process of retrodifferentiation but, in any case, could have profound clinical and pharmacological implications. Finally, the flexibility and the speed by which a variety of stem cell classes can be generated *ex vivo* from donor blood could potentially transfer this novel process into a less invasive automated clinical procedure.

Introduction

Somatic cell plasticity is an emerging field in stem cell biology. In this respect, a variety of more committed cells have been shown to re-switch their developmental potential in response to either nuclear transfer into an enucleated oocyte[1], or to more remote micro-environmental cues or pressures, as is the case with stem cells *in vivo*[2–5]. On the other hand, the phenomenon of cell transdifferentiation, during which more committed cells traverse the differentiation barrier and adopt a new specialisation fate *in vitro*, is well documented in the literature.

Transdifferentiation from one specialised fate to another has been demonstrated for many cell types: retinal pigmented epithelial cells into lens tissue or

retinal neuronal cells[6,7]; keratinocytes into mesenchymal myogenic-like cells[8]; dermal papilla cells into hair follicle epidermal cells[9]; squamous vaginal epithelial cells into cuboidal mucinous cells[10]; human pancreatic islet cells to pancreatic ductal cells[11]; fat storing cells into myofibroblasts[12]; chondrocytes into osteocytes[13]; oligodendrocyte precursors into multipotential CNS stem cells[14]; blood cells into brain[4] and vice versa[5] and pro-B cells into a variety of leukocyte subsets[15]. In most cases, the newly generated cells have been noted to redifferentiate into cells of the original lineage and stage.

While transdifferentiation is well documented, the underlying mechanisms remain poorly understood. The loss of differentiation markers, commonly termed dedifferentiation[16], has been demonstrated in multinucleated heterokaryons[17], a transfected cell line[18], gut cells[19] and mammary epithelial cells[1]. However, explanations for dedifferentiation provided to date are ambiguous because they do not elucidate what is behind the loss of the differentiated state. Analyses of *de novo* gene activation in such cells suggest some sort of reprogramming.

Another mechanism proposed to explain the partial loss of the differentiated state of a cell is retrodifferentiation[20-25] which is defined in terms of what occurs during forward differentiation of a precursor cell. The principle underlying retrodifferentiation is the inversion of the differentiation programme to generate a cell at a progenitor or stem cell stage. In this form of retrograde development, a committed cell reverts to an earlier ontogenic stage. In contrast to transdifferentiation, evidence supporting retrodifferentiation is meagre, coming primarily from studies of cell lines such as myelomonocytic[26] and erythroid[27] leukaemias, regenerating liver cells[28] and neoplastic colon cells[29]. Such studies previously reported loss of differentiation markers, however, not to a stem cell stage.

Pluripotent stem cells with multi-developmental potentials are not found circulating in unmobilised adult human peripheral blood. These types of precursors have been reported recently to reside in the bone marrow[30]. The wide clinical indications where stem cell therapies are believed to benefit many patients with leukaemia, lymphoma, some solid tumours or degenerative diseases puts an added constraint on their current sources. Unmobilised adult peripheral blood contains insufficient quantities of stem cells to be used to treat such a wide spectrum of clinical indications. Herein, I report the *in vitro* production of a variety of stem cell classes derived from mononuclear cells (MNC) obtained from human adult peripheral blood in response to the addition of purified CR3/43 monoclonal antibody (mAb) to well-established culture conditions.

Materials and methods

Cell Culture

Haematopoietic-Conducive Conditions (HCC)

MNC were obtained from healthy human buffy coat samples (obtained from the National Blood Service, Brentwood, England) by density gradient centrifugation on Histopaque (Sigma) at a specific gravity of 1.077 *g*. After washing, MNC were resuspended at 2×10^6 per ml in Dexter's long-term culture (LTC) medium[31] consisting of Iscove's Modified Dulbecco's Medium (IMDM) without phenol red (Invitrogen), 10% foetal calf serum (FCS), 10% horse serum (HS) (Sigma), 10^{-7} M cortisol (StemCell Technologies), and 1% penicillin/streptomycin (Sigma) supplemented with 3.5 µg per ml of purified CR3/43 (DakoCytomation). This constituted the haematopoietic-conducive condition (HCC).

The CR3/43 clone was generated by DakoCytomation in the absence of azide and antibiotics. The CR3/43 mAb is raised against human monomorphic regions of the beta chain of the major histocompatibility complex (MHC) class II antigens DP, DQ and DR. This antibody binds to B cells, monocytes, antigen-presenting cells and activated T cells. Alternatively, the animal sera, cortisol and antibiotic components of the HCC culture medium can be replaced by citrated human autologous plasma (ACDA, Baxter Inc.) supplemented with 7 µg/ml CR3/43. In order to increase the yield of $CD34^+$ cells, human leukocytes enriched by dextran sedimentation or aphaeresed mononuclear fractions can be used instead of histopaque-separated MNC. Cells were plated in six-well plates (Fisher Scientific, USA) at 2 ml per well and incubated at 37 °C and 5% CO_2 in air. The cells were fed the following day and, thereafter, every 4 days with Dexter's medium in the absence of CR3/43.

Neuronal-Conducive Conditions (NCC)

MNC were prepared and seeded as described above except that these were cultured in embryonic stem (ES) culture media[31] consisting of Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen), 20% FCS (Sigma), 1% L-glutamine (Sigma), 1% MEM non-essential amino acids (Invitrogen) and 0.2% of 0.1M β-2-mercapto-ethanol (Sigma). Cells were fed the following day and thereafter every 4 days with ES medium in the absence of CR3/43.

Cardiac Conducive Conditions (CCC)

MNC were obtained as above and seeded as 20 µl 'hanging drops'[33] in ES[32] or LTC culture media[31], as detailed above, containing 3.5 µg/ml purified CR3/43. Alternatively, MNC were subjected to HCC or NCC, minus cortisol and β-2-ME respectively, and seeded at

2×10^6 per ml in six-well plates (Fisher Scientific USA) and chamber slides coated with 0.1% gelatin (StemCell Technologies). Beating areas in hanging drops[39] were observed using an inverted phase contrast microscope (Olympus CK-40) and imaged using a digital video camera.

Clonal Assays

Cells were seeded in methocult GFH4434 according to manufacturer instructions (StemCell Technologies) containing recombinant human growth factors. Differentiation into haematopoietic cell colonies was assessed and colonies were inspected and scored with time using phase contrast microscopy (Olympus CK-40).

Purification of CD34 Cells by Positive Selection

Twenty-four hours post-CR3/43 treated MNC under HCC were purified using the CD34 MultiSort Kit (Miltenyi Biotec) according to manufacturer's instructions. Briefly, prior to isolation, cells were subjected to Fc receptor blocking to prevent non-specific binding followed by direct labelling of cells with anti-human class II CD34-coated microbeads. The labelled cells were passed twice through an LD Midimacs separation column in the MACS separator and bound cells were gently flushed and collected for analysis.

Purification of Undifferentiated Cells by Negative Selection

Twenty-four hours post-incubation, an aliquot of cells incubated under NCC were subjected to co-negative selection using anti-CD45 and anti-glycophorin A-coated magnetic microbeads and applied to the LD Midimacs column in the MACS separator all according to the manufacturer's instructions (Miltenyi Biotec). The unbound cells were retained, passed through a further magnetic separation step and collected as the CD45-negative/glycophorin A-negative population for analysis.

Confocal Microscopy

In order to facilitate live imaging by confocal microscopy, cells cultured in HCC were plated in organ culture dishes whereby a cover slip formed an integral part of its base. Conjugated anti-human antibodies against CD19-fluorescein isothyocyanate (FITC) and class III CD34-R-phycoerythrin-Cy5 (RPE-Cy5) (both DakoCytomation) were added at the recommended dilutions directly to the reaction/cell mixture. Cells were imaged every 3 min for up to 12 h at room temperature. Imaging of colonies in methocult culture was performed once colonies had reached maturity by directly adding the following fluorescent conjugated anti-human antibodies to the culture media: glycophorin A-FITC; CD33-RPE-Cy5; CD61-FITC (all DakoCytomation).

Flow Cytometry

Cultured cells harvested at specified time points were washed and resuspended in PBS containing 10% human AB serum (Sigma) or an Fc blocking reagent (Miltney Biotec) to block non-specific binding. Due to homotypic aggregation and adhesion induced in response to treatment with CR3/43, single-cell suspensions were obtained by continuous scraping, stirring and pipetting of the cultured cells. According to the manufacturer's instructions, cells were labelled for 15 min at 4°C with the following directly-labelled anti-human antibodies: class III CD34-PE, CD34-FITC and CD34-RPE-Cy5, CD38-PE, CD38-FITC, CD45-FITC, c-kit-PE, CD33-PE, CD61-FITC, glycophorin A-PE, CD19-PE and CD3-FITC (all DakoCytomation) and CD133-PE (Miltenyi Biotec). Autofluorescence and negative controls were determined throughout; isotype negative controls IgG1-FITC, IgG1-PE and IgG1-RPE-Cy5 (all DakoCytomation) were used. Cells were washed with cell wash (Becton Dickinson). Events ranging from 20 to 100 000 were acquired using FACScan (Becton Dickinson) and analysed using Cellquest software version 3.3.

Formation and Immunohistochemical Staining of Embryoid Body (EB)-Like Structures

Twenty microliters containing 300 purified cells (as described above for negative selection) (i.e. 1.5×10^4 cells per ml) were resuspended as 'hanging drop' cell cultures in ES medium[39]. EB-like structures were plucked from 'hanging drop' cultures and transferred to microscope slides pre-coated with poly-lysine. A coverslip was applied and the slides were incubated at –80°C for 15 min prior to fixing. In contrast, MNC cultured in NCC and CCC were directly fixed in chamber slides prior to blocking and staining. All samples were fixed for 15 min in 4% paraformaldehyde (Sigma). EB-like structures were stained using HRP-conjugated antibody for analysis by phase contrast microscopy. For HRP-conjugated staining, fixed embryoid body (EB)-like structures were incubated with peroxidase block (DakoCytomation) for 5 min and then further blocked and permeabilised using 0.15% Triton X-100/10% rabbit serum/PBS. Thereafter, EB-like structures were independently incubated with one of the following primary anti-human monoclonal antibodies: myocardial-specific actin; alpha-fetoprotein; cytokeratin-7 and -20; Desmin; S100 (all Dakocytomation) and pan-neurofilament (Sternberger Monoclonals) and then detected using a rabbit anti-mouse HRP (DakoCytomation), with all antibody dilutions recommended by the manufacturer. Nuclei were stained with haematoxylin (DakoCytomation). Faramount medium (DakoCytomation) was used for mounting and

the stained cells visualised by inverted phase contrast microscopy (Olympus CK-40). Imaging was subsequently performed using a digital camera attached to the microscope. Cystic formation by embryoid body was performed by staining suspension of intact cell clusters with DiI-C18 (kind gift from Dr Tim McCaffery). Following copious washing, embryoid bodies were analysed using fluorescence microscopy.

Immunohistochemical Staining of Neuronal and Cardiomyogenic Development

MNC cultured in NCC and CCC were fixed as above, blocked using 0.15% Triton X-100/10% donkey serum/PBS and then differentially co-stained for neuronal and cardiac-specific markers respectively. For neuronal-specific staining, cells were co-stained with NF/GFAP, MAP-2/Tau or oligodendrocyte/CD45-FITC primary conjugate (DakoCytomation). In this case, primary anti-human monoclonal antibodies against pan-NF (Sternberger Monoclonals), MAP-2 (Sigma) and oligodendrocyte (Chemicon International) were detected using donkey anti-mouse RPE-Cy5 (Jackson Immunoresearch) whilst second-stage rabbit anti-human GFAP (DakoCytomation) and Tau (Chemicon International) were detected using donkey anti-rabbit FITC (Jackson Immunoresearch) and CD45 was directly stained with anti-human CD45-FITC conjugate (DakoCytomation).

MNC cultured in CCC were differentially co-stained for either cardiac-specific troponin I or myocardial-specific actin and human CD45. In this case, the primary anti-human monoclonal antibodies against troponin I (Fitzgerald Industries Inc.) and actin (Dakocytomation) were both detected using donkey anti-mouse RPE-Cy5 (Jackson Immunoresearch) whilst second-stage anti-human monoclonal antibody against CD45 was directly conjugated to FITC. In all cases, nuclei were stained using propidium iodide (PI) (Sigma). In addition, human NF, MAP-2 and GFAP were detected using TRITC-labelled rabbit anti-mouse conjugated antibody and nuclei stained with hoechst. Imaging was subsequently carried out by confocal microscopy.

Reverse-Transcriptase PCR Analysis

Total RNA was isolated from cells at recorded time intervals using the RNAzol reagent (Biogenesis) according to the manufacturer's instructions. The RNA was reverse transcribed using Moloney murine leukaemia virus reverse transcriptase (Promega). The reverse transcriptase products served as a template for independent PCR reactions using the thermostable Taq polymerase (Promega). For PCR analysis, OCT-4[34], nestin[35], CD34[36], GATA4, hANP and cTnT[37] primers were used.

Results

Haematopoietic Analyses

Live Image Analysis using Confocal Microscopy

Treatment of MNC obtained from adult peripheral blood with purified CR3/43 mAb increases the relative number of CD34$^+$ cells cultured in Dexter's LTC medium[31]. The CR3/43 mAb is raised against human monomorphic regions of the beta chain of the major histocompatibility complex (MHC) class II antigens DP, DQ and DR. This antibody binds to B cells, monocytes, antigen-presenting cells and activated T cells. Under such HCC, live imaging of MNC using confocal microscopy (Figure 1A–L) reveals upregulation of the haematopoietic stem cell marker, CD34, concomitant with downregulation of the mature B lymphocyte marker CD19 (see supplementary data – real-time movie A[38]). These phenotypic changes occur within an hour of adding CR3/43 to the MNC cultured in Dexter's medium and are accompanied by cell motility in the culture dish.

Flow Cytometry Analysis

Immunophenotypic analysis of MNC under HCC before and after 2 h and 24 h treatment with CR3/43 mAb (Figure 2) shows significant increase in the relative number of cells expressing CD34 in response to treatment. The majority of CD34$^+$ cells are CD45 low and either positive or negative for CD38 antigen, typical of committed and more primitive haematopoietic progenitor cells[39,40], respectively. The latter type has been shown to possess more long-term SCID repopulating potentials[39]. By 24 h in HCC, a significant proportion of CD34$^+$ cells co-express c-Kit or CD133 (Figure 2A). Furthermore, significant numbers of CD34$^+$ cells were purified from MNC cultured for 24 h in HCC, using well-established haematopoietic progenitor purification procedures[41] such as magnetic beads labelled with anti-human CD34 (Figure 2B). The purified CD34$^+$ cells exhibited, as for conventional haematopoietic stem cells, CD45 at low levels with or without CD133. The CD34$^+$ CD133$^+$ cells were more CD34 bright[42] than those cells that were CD34$^+$CD133$^-$. A significant proportion of CD34$^+$ cells were either CD38 positive or negative. The latter immunophenotype have been reported to possess more long-term SCID repopulating potential[39] Moreover, significant levels of CD34 transcripts[36] were amplified from the purified CD34$^+$ cells and unfractionated MNC cultured for 24 h in HCC when compared to MNC cultured in LTC alone (Figure 2C).

Figure 1. (Continued on pages 360 and 361)

Figure 1. (Continued)

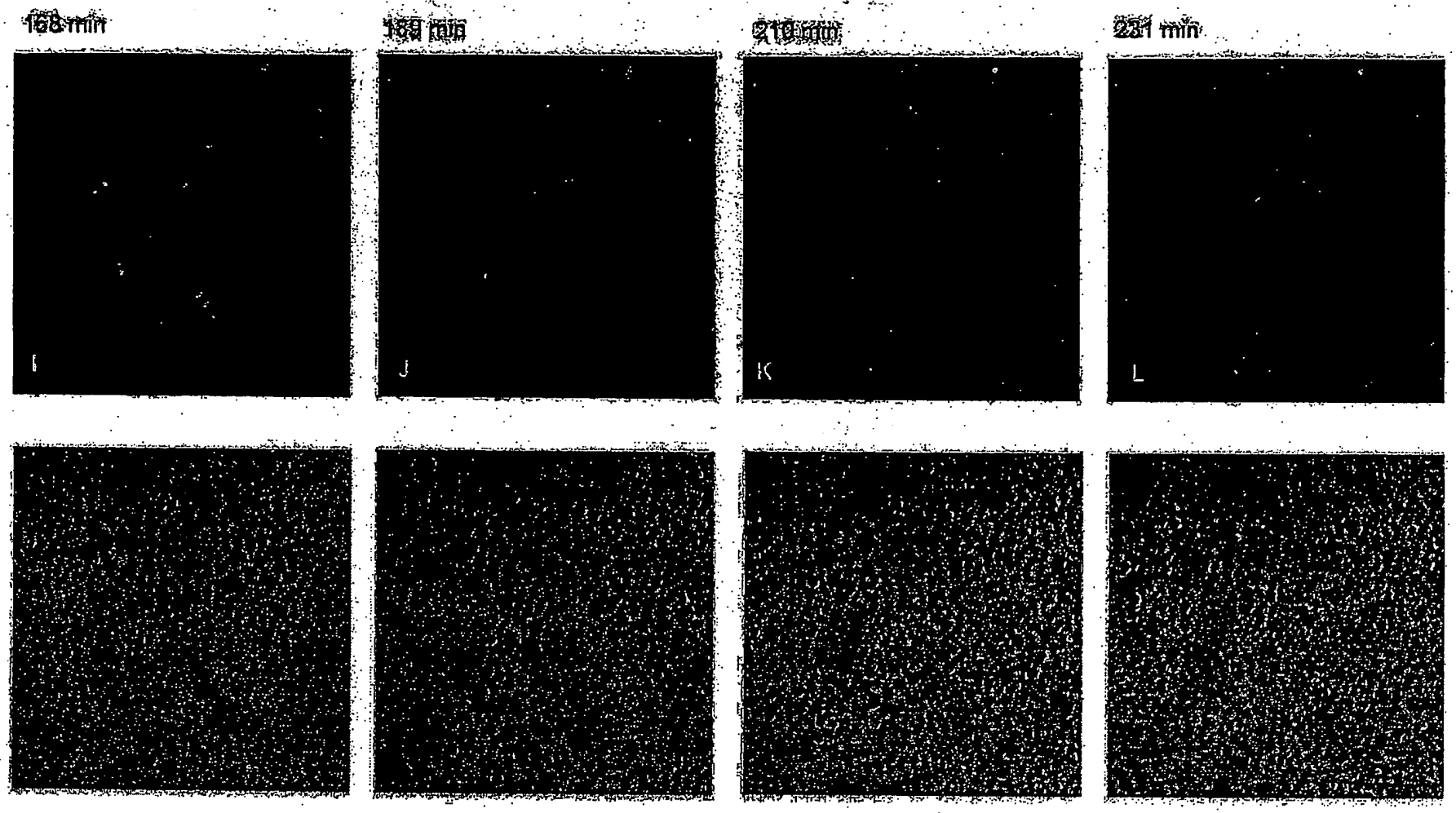
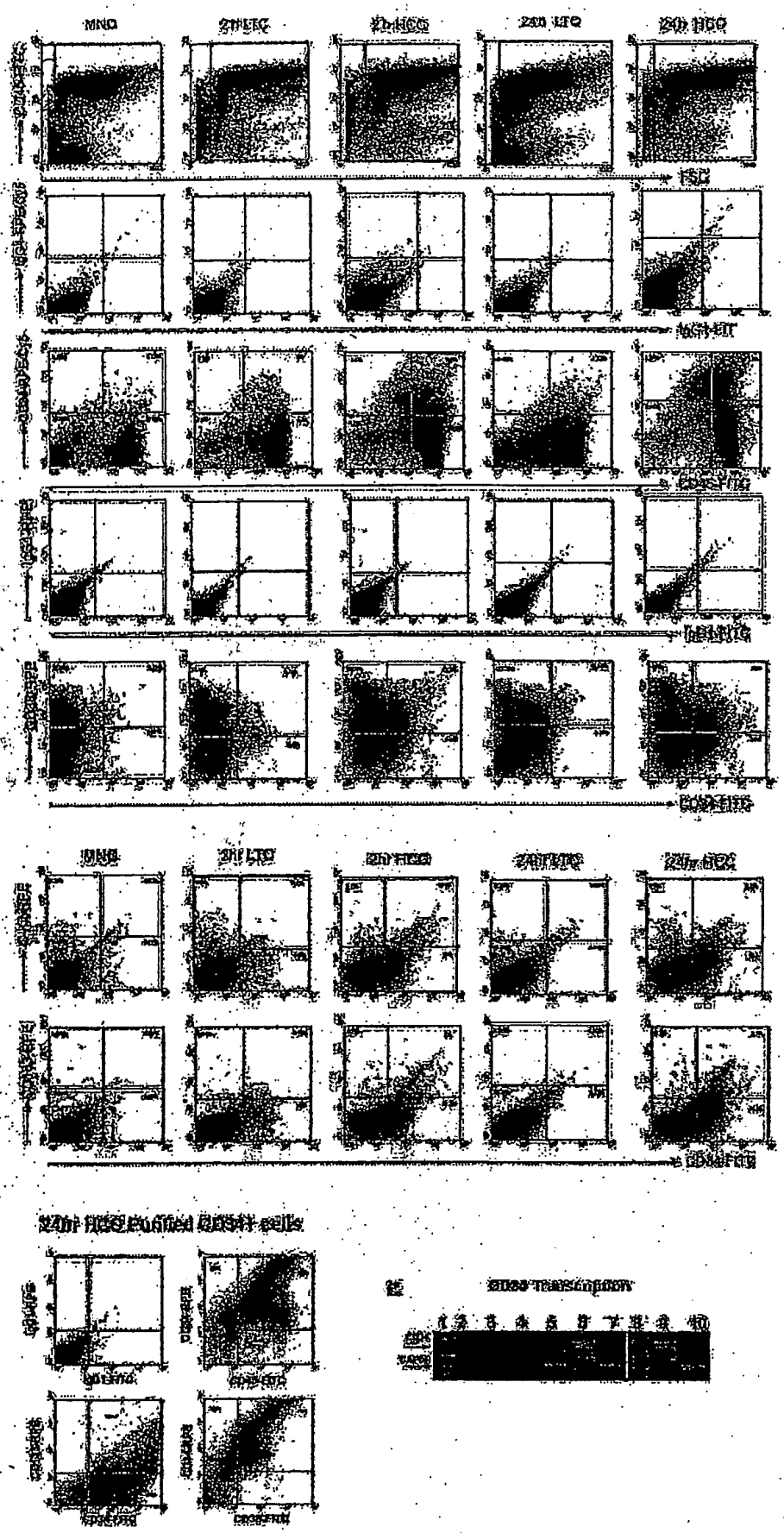


Figure 1. Confocal microscopy showing the upregulation of CD34 concomitant with the down regulation of CD19 by MNC cultured in HCC. Cells were live imaged every 3 min for up to 12 h. Anti-human CD19-FITC (green) and CD34-RPE-cy5 (red) antibodies were added to the cell mixtures directly in order to visualise changes in cell receptor expression. Frames (A–L) are selected at 21-min intervals. Frame (A) and (B–L) are MNC before and after addition of CR3/43 mab to LTC respectively. Lower panels show the corresponding phase contrast image of the upper panels at the same time points. In addition, the superimposed real-time movie of the above images shows the movement of cells during up-regulation of CD34 (refer to Real-time movie A[11])



Following cryopreservation of the 24 h MNC cultured in HCC and subsequent purification of $CD34^+$ cells, the yield of viable $CD34^+$ cells per one unit of buffy coat (500 ml of donated blood) as measured by flow cytometry, and cell viability assessed by trypan blue dye exclusion assay, is approximately $100–150 \times 10^6$ cells and dependent on MNC input. For example, higher yields of MNC are obtained via aphaeresis or dextran sedimentation than when fractionated on a density gradient. Throughout culturing of MNC in HCC, cells remain viable and there is an approximate 1.6-fold increase in their absolute numbers 24 h later. On the other hand, 24 h culturing of MNC in LTC (not supplemented with CR3/43 mAb) gives rise to an increase (about 20%) in the number of erythrocytes and dead cells (Figure 2). Viability assessment of MNC cultured in HCC or LTC at 2 h and 24 h from culturing following cryopreservation, showed 30% cell death in cells cultured in LTC compared to 5% in the HCC group (data not shown).

Colony Formation

By 2 h in HCC, MNC were rendered clonogenic when single-cell suspensions were seeded in methocult-containing growth factors. They produced a variety of haematopoietic colonies such as colony-forming unit-granulocyte, erythroid, monocyte, macrophage, megakaryocyte (CFU-GEMM), colony-forming unit-granulocyte, monocyte, macrophage (CFU-GM), colony-forming unit-monocyte, macrophage (CFU-M), blast-forming unit-erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E) (Figure 3). On the other hand, seeding MNC, cultured for 24 h in HCC, at the same cell density in methocult medium (basic or growth factor-containing) supplemented with 300 µl of 24 h HCC supernatant (to basic or growth factor-containing medium) resulted in the formation of much larger and wider variety of colonies, including megakaryocytes, indicating the secretion of cytokines by cells cultured in HCC (Table 1). On the other hand, the clonogenic efficiencies of purified $CD34^+$ cells obtained from MNC cultured for 24 h in HCC is 1 in 2.5 when compared to a value of 1 in 10^5 MNC cultured for 24 h in LTC (data not shown). Direct immunostaining of these high proliferative potential[43] (HPP-CFC) haematopoietic colonies in methocult cultures with anti-human antibodies specific for myeloid and erythroid antigens using confocal microscopy showed differential expression of glycophorin A without CD33, CD33 without CD61 and CD33 with CD61 (Figure 3R–T), a staining pattern typical of erythrocytes, monocytes/granulocytes and megakaryocytes, respectively. More significantly, ploidy or cell cycle analysis of fully differentiated haematopoietic colonies grown in methocult show normal DNA content (data not shown). Furthermore, within 24 h, MNC cultured in HCC gave rise to cobblestone areas and stromal-like cells (Figure 3K). In addition, cryopreserved 2 h and 24 h MNC in HCC were clonogenic when compared to the same MNC population cultured in LTC. Interestingly, the time at which MNC became capable of repopulating non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice[44] at a relatively higher level and frequency was 3 h following the addition of purified CR3/43 mAb to MNC cultured in Dexter's medium (manuscript in preparation).

Purification and Characterisation of Undifferentiated Cells

Apart from red blood cells, leukocytes and extremely low levels of haematopoietic precursors, mature healthy blood is not known to contain undifferentiated cells or neuronal precursors. Treatment of MNC cultured in ES culture media[32] with 3.5 µg/ml purified CR3/43 mAb resulted, by 24 h, in an increase in the number of undifferentiated cells that were both CD45 and CD34 negative (Figure 4A). These cells were purified by negative selection on a Midi Macs column using anti-human CD45

Table 1. Percentage of clonogenic progenitors within MNC cultured in HCC after 24 h (324 ± 37.9 colonies derived per 1×10^4 nucleated cells)

CFU-GEMM (%)	CFU-GM (%)	CFU-M (%)	BFU-E (%)	CFU-Meg (%)
47.9 ± 5.8	19.8 ± 3.9	5.4 ± 2.2	9.7 ± 2.0	16.0 ± 3.1

CFU-GEMM indicates colony-forming unit (CFU)-granulocytic, erythroid, monocyte, macrophage, megakaryocytic; CFU-GM, CFU-granulocytic, monocyte, macrophage; CFU-M, CFU-monocyte, macrophage; BFU-E burst-forming unit-erythroid and CFU-Meg, CFU-megakaryocytic. The percentage of each colony type is calculated as the mean ± standard error of mean (SEM) ($n = 5$). The number of phenotypically distinct progenitors were identified and measured from the use of plating conditions according to standard protocols

Figure 2 (opposite). Increase in the number of $CD34^+$ cells in response to MNC cultured in HCC (λ). Cells were stained as indicated with a panel of conjugated monoclonal antibodies against human CD34, CD45 and CD38, c-kit and CD133 antigens before and after (2 h and 24 h) culturing MNC in HCC (Dexter's medium with CR3/43 mab) and LTC (Dexter's medium alone). Cells analysed by FACScan were gated according to FSC and CD45 and in conjunction with the relevant isotype negative control. Purified $CD34^+$ cells from 24-h MNC cultured in HCC using the CD34 MultiSort Kit (B). Cells were stained with a panel of conjugated monoclonal antibodies against human CD34 and CD45, CD34 and CD38 and CD34 and CD133. (C) RT-PCR showing CD34 transcription by purified and unfractionated 24 h MNC cultured in HCC. Lanes 1 and 8, 1 KB ladder; lanes 2 and 3, GAPDH and CD34 gene transcript negative controls, respectively; lanes 4 and 5 are CD34 and GAPDH gene transcripts, respectively, in 24 h MNC/LTC; lanes 6 and 7 are CD34 and GAPDH gene transcripts, respectively, in purified $CD34^+$ cells from 24 h MNC/HCC; lanes 9 and 10 are CD34 and GAPDH gene transcripts, respectively, in unpurified 24 h MNC/HCC

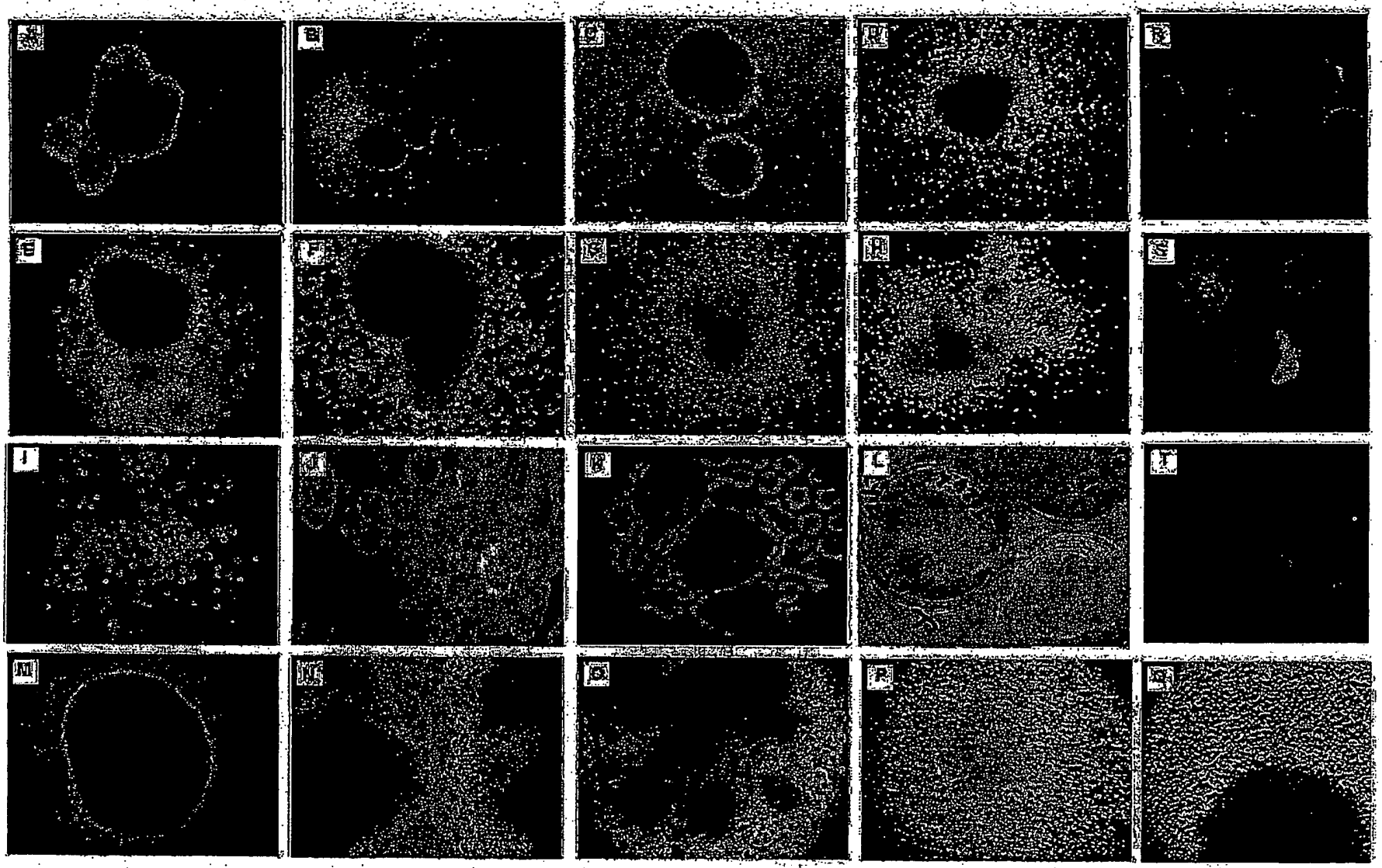
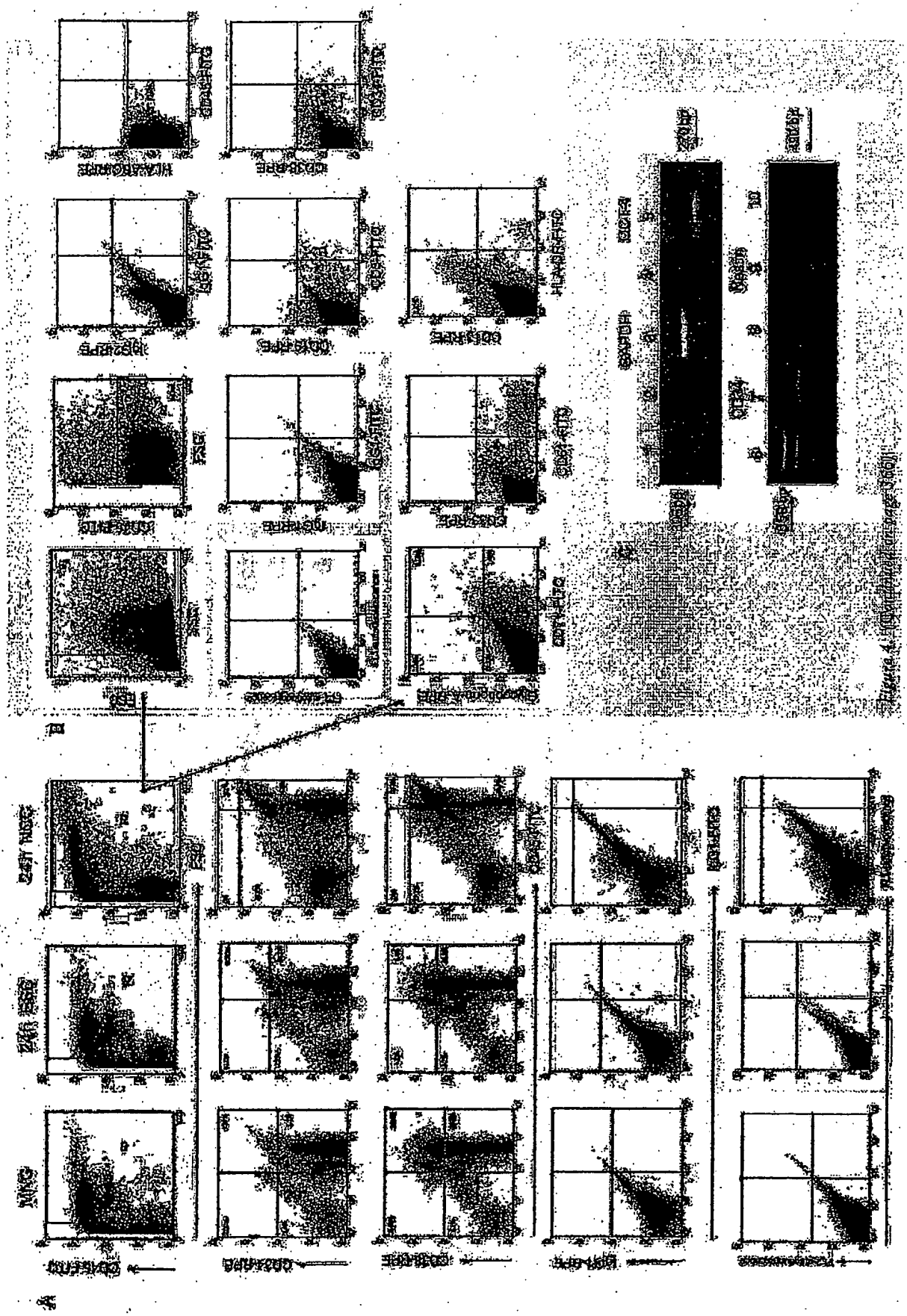


Figure 3. Different types of clonogenic cells are produced in response to treatment of MNC in (A–K) HCC and (L–Q) NCC. Panels: (A–C) represent BFU-E; (D–F) CFU-GEMM; (G, H) CFU-GM; (I) CFU-M; (J) CFU-Meg; (K) cobble stone areas and stromal like cells. In contrast, MNC cultured in NCC gives rise to colonies that resemble (M) embryoid bodies or (N, O) large erythroid or (P, Q) myeloid-containing colonies. Such large colonies are (L) visible to the naked eye. In addition, differential co-staining of colonies produced by MNC cultured in HCC demonstrates (R) glycophorin A-positive (green) and CD33⁻ (red) (S) CD33 (red) and CD61 (green) co-positive and (T) CD33⁺ (red) and CD61⁻ (green) haematopoietic lineages typical of erythroid, megakaryocytic and granulocytic/monocytic cell lineages, respectively



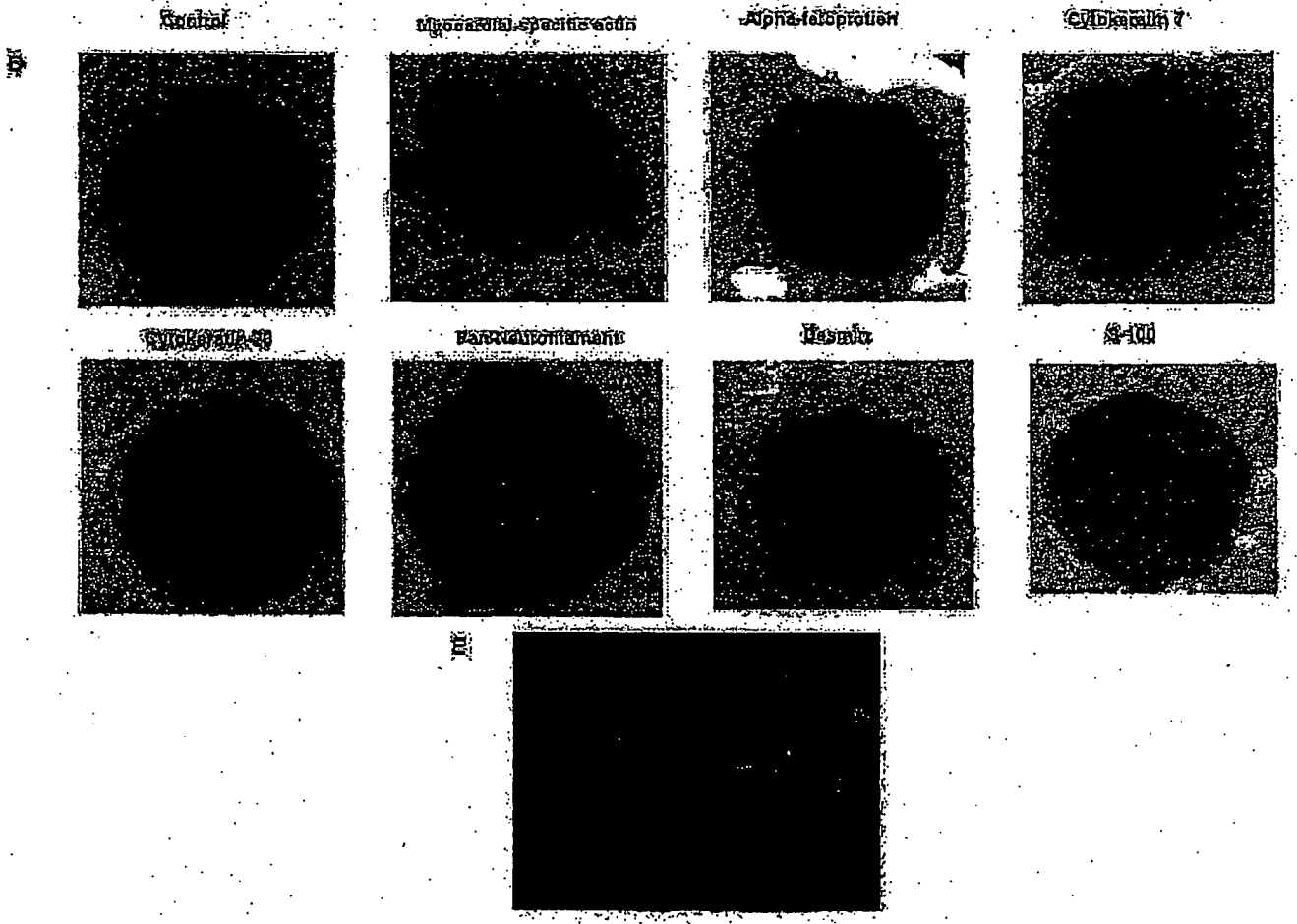


Figure 4. Increase in the numbers of undifferentiated CD45/lineage negative cells in response to MNC cultured in ES medium and supplemented with CR3/43 mab. For (A, B) FACScan analysis, 24-h MNC cultured in ES medium with or without CR3/43 mab were harvested and stained as indicated with a panel of conjugated monoclonal antibodies against human (A) CD34, CD45 and CD38 antigens. (B, C) Characterisation of the purified undifferentiated CD45/lineage-negative cells by flow cytometry and RT-PCR, respectively. In (B) flow cytometry shows the purified cells are HLA-ABC-CD45-CD19-CD3-CD34-CD38-CD71-glycophorinA-CD33-CD61-CD13-HLA-DR negative. In (C) RT-PCR shows that the purified cells transcribe high levels of (lanes 3 and 5, respectively) GAPDH and OCT-4 and to a lesser extent (lanes 7 and 9 respectively) CD34 and nestin. Lanes 1 and 6 are 1 KB ladder, lanes 2, 4, 8 and 10 are negative controls for GAPDH, OCT-4 CD34 and nestin, respectively. (D) Immunohistochemical analysis of EB-like structures formed 48 h following culturing of the purified cells (characterised in B and C) as 'hanging drops' (resuspended as 300 cells per 20 µl drop) show that they are positive for myocardial-specific actin, alpha-fetoprotein, cytokeratin 7 and 20, pan-NF, desmin and S-100 (brown stain) when compared to negative control EB-like structures stained with HRP conjugated secondary antibody alone. Nuclei were stained blue with haematoxylin. The stained cells were visualised by inverted phase contrast microscopy (Olympus CK-40). Imaging was subsequently performed using a digital camera attached to the microscope. (E) DiI-C18 labelled cystic embryoid body

and glycophorin A-coated magnetic beads (Miltenyi Biotec), in order to deplete leukocytes and red blood cells, respectively. The unbound cells were analysed by flow cytometry using a panel of anti-human monoclonal antibody conjugates. The purified cells were negative for haematopoietic-associated markers that are typically expressed by the erythroid, lymphoid and myeloid cell lineages and include MHC class I and II antigens (Figure 4B). In addition, such purified cells transcribed high levels of the embryonic stem cell marker, OCT-4, and, to a lesser extent, nestin and CD34 (Figure 4C). Following purification, 95% of these cells were viable and, moreover, one unit of buffy coat can yield up to 90–130 × 10^6 of such undifferentiated cells.

Upon culturing as 'hanging drops'[33], the purified cells formed cystic EB-like structures by 48 h that expressed ectodermal, endodermal and mesodermal antigens including alpha-fetoprotein (Figure 4D–E). Taken together, these analyses demonstrated the production of undifferentiated cells 24 h following the addition of CR3/43 mAb to MNC cultured in ES medium. Similarly, 24 h of culturing MNC in ES medium containing CR3/43 grown either on chamber slides (StemCell Technologies) or six-well plates resulted in the formation of EB-like structures that are loosely attached to the substratum and stain positive for ectodermal, mesodermal and endodermal antigens[45,46]. In addition, single-cell suspensions obtained from 24 h MNC cultured in ES medium containing CR3/43 mAb seeded in methocult medium (containing recombinant growth factors and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 and 20% O_2) produced uniform colonies akin to embryoid bodies (Figure 3M). In contrast the same cells, seeded in the same methocult at 37 °C containing 5% CO_2 and 5% O_2, formed larger colonies visible to the naked eye (Figure 3L) consisting of myelocytes and erythrocytes or myelocytes only (Figure 3, N–O and P–Q, respectively).

Neuropoietic Analyses

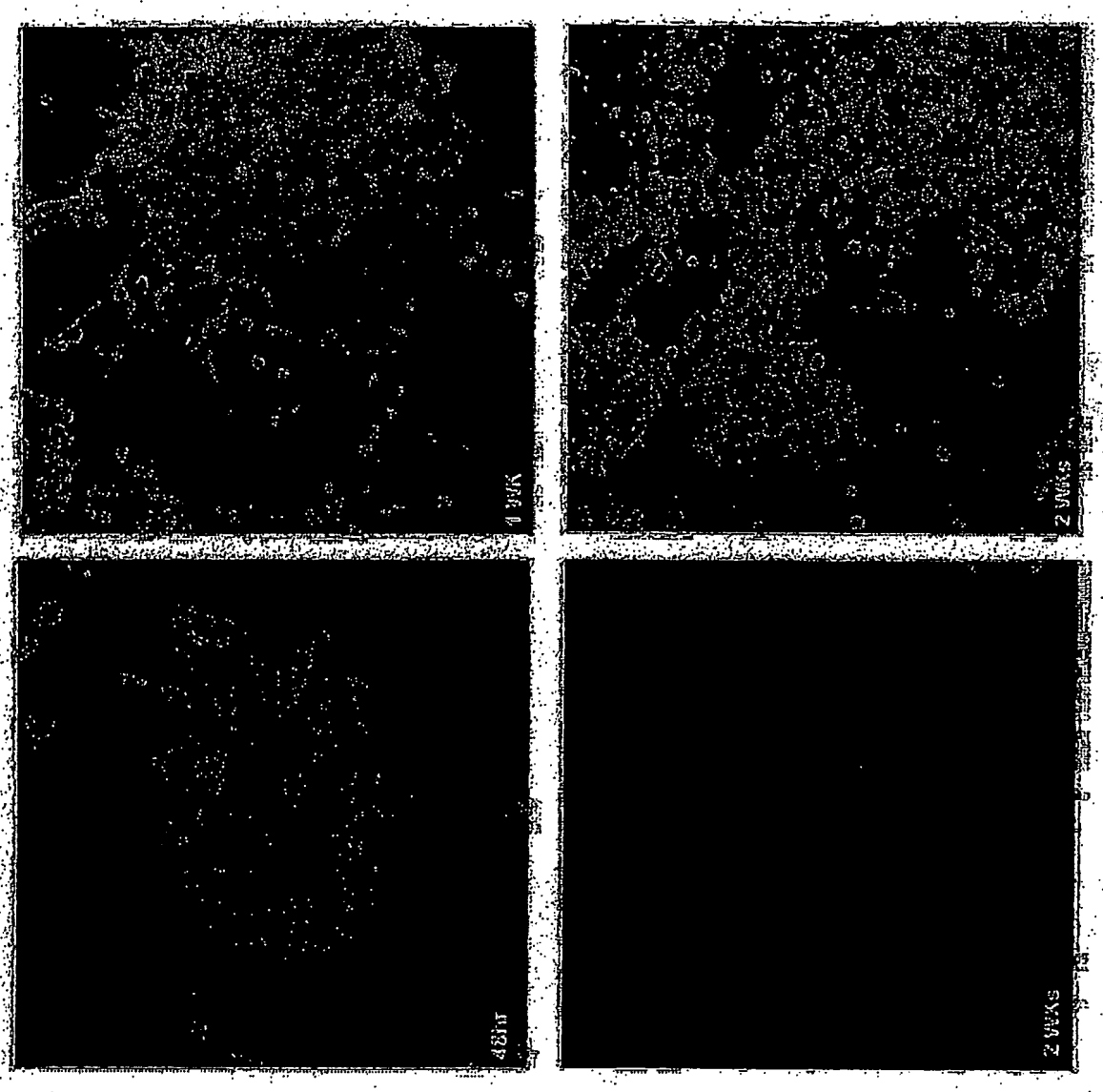
Notably, continued culturing of MNC in ES medium initially containing CR3/43 mAb resulted in the eventual conversion of MNC into spherical bodies and neuronal-like cells (Figure 5A). By 24 h, MNC in NCC, transcribed OCT-4 and nestin (Figure 5B)[34,35] and on maturation stained positive for MAP-2, glial fibrillary acidic protein (GFAP) and neurofilament (NF) 200 and 70 by weeks 1 and 2 (Figure 5C). This process was always accompanied by downregulation of nestin and OCT 4.

Dual immunohistochemical staining of MNC in NCC performed at defined time points using antibodies to MAP-2 and Tau, NF and GFAP and oligodendrocyte and CD45, and analysed by confocal microscopy, showed differential expression of neuronal, glial and oligodendrocytic antigens[47]. For example, by 48 h, cellular spheres formed that co-stained positive for MAP-2 and Tau, indicative of neurospheres containing immature neurons. One and 2 weeks later, the spheres differentiated into mature neurons, with MAP-2 being confined to cell bodies and Tau to the axons (Figure 5D). On the other hand, spheres that were analysed for NF and GFAP showed the presence of neuronal and glial precursors. However, 1 and 2 weeks later, such spheres differentiated into neurons expressing NF alone without GFAP, or astrocytic-like cells expressing GFAP without NF, the latter typical of glial cells (Figure 5E). In contrast, co-staining for the pan-leukocyte marker, CD45, and oligodendrocyte, indicated the absence of CD45 expression at all time points shown, while oligodendrocyte staining was confined to the periphery of the spheres and persisted in 1- and 2-week-old cultures (Figure 5F). Moreover, time course analysis of neurotransmitters released by these neurons in response to depolarisation revealed that, as they matured, the neurons produced increasing amounts of glutamate, GABA, tyrosine, dopamine and serotonin and uptake of taurine (unpublished data).

Cardiopoietic Analyses

The cardiomyogenic-conducive condition (CCC) involves culturing of MNC either as 'hanging drops'[20] or in HCC or NCC in six-well plates or chamber slides, coated with 0.1% gelatin. In 'hanging drops', MNC cultured in CCC formed compact embryoid-like bodies that started to beat in an asynchronous manner within 24 h from the initiation of such cultures. These large masses of cell aggregates were surrounded by novel single cells that aligned themselves along parallel axes while undergoing synchronous cyclical beating rhythms consisting of torsional contraction, elongation and rotation (see supplementary data – Real-time movie B[48]). These novel cells were found in the meniscus at the centre of the hanging drops. Interestingly, the relative number of contracting cells or embryoid-like structures in the 'hanging drops' increased significantly when cortisol and mercaptoethanol were removed from HCC or NCC, respectively. Moreover, azide was noted to be a powerful inhibitor of the beating cells. Within 1 week, MNC in CCC grown on gelatinised six-well plates or chamber slides transcribed the cardiac transcription factor GATA-4, human atrial natriuretic peptide (hANP) (Figure 6B) and cTnT[37]. Dual immuno-histochemical analysis of 1-week-old CCC-cultured MNC revealed the expression of myocardial-specific actin and cardiac-specific troponin I without CD45 expression (Figure 6A). More interestingly, 3-day and 7-day-old MNC cultured in CCC were able to differentiate into fully mature cardiomyocytes when infused into the myocardium of non-irradiated non-infarcted *Rnu/Rnu* nude rats[49] (unpublished data).

Figure 5. (Continued on pages 369–371)



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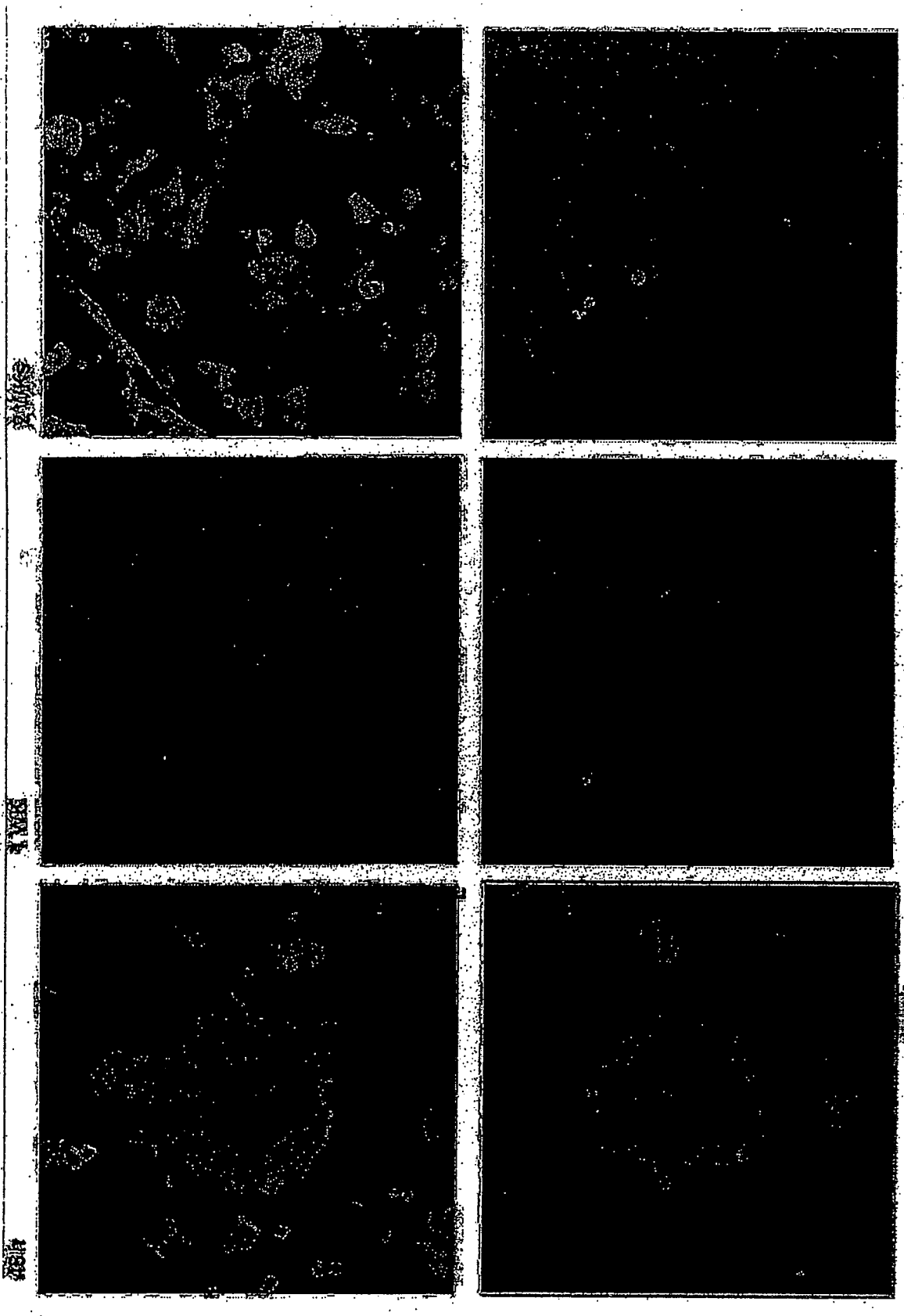

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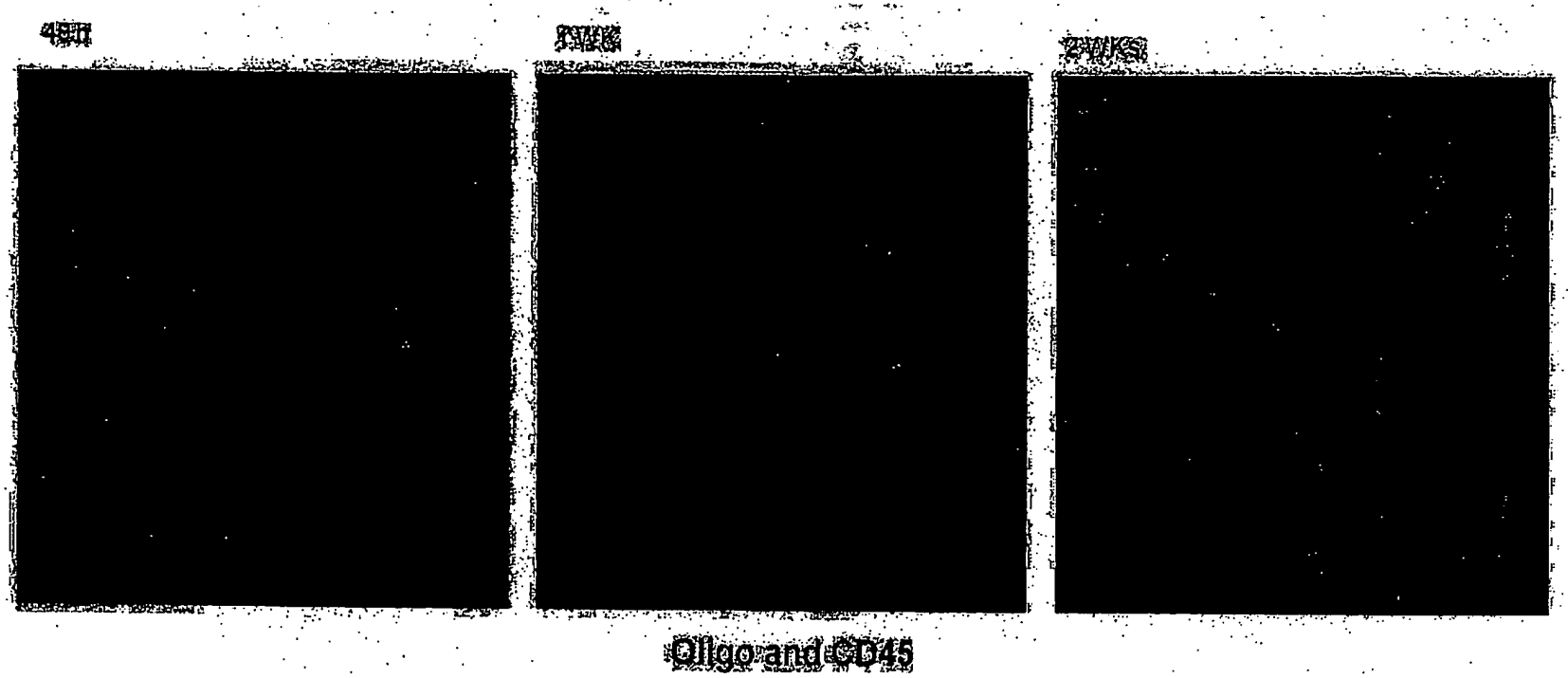

Figure 5. Time course analysis of neuronal and non-neuronal differentiation (A) morphological (B) genetic and (C) phenotypic changes in MNC cultured in NCC and grown on chamber slides or six-well plates. (A) Phase contrast microscopy of MNC before and after 24 h, 1, 2 and 3 weeks in NCC demonstrates the formation of spherical structures and progressive development of cells with neuron-like projections. (B) RT-PCR analysis of OCT-4, nestin and positive control GAPDH-specific transcript expression. Lane orders for each gene transcript; lane 1, 100-bp ladder, lane 2, gene transcript-specific negative control, lane 3, 24 h MNC/NCC; lane 4, 1 week MNC/NCC; lane 5, 2 weeks MNC/NCC; lane 6, 3 weeks MNC/NCC and lane 7, MNC alone. (C) Confocal microscopy of NCC-cultured MNC at defined time points co-stained with the nuclear stain hoechst (blue) and either NF, GFAP or MAP2 (each red) demonstrating the progressive acquisition of neuronal-specific markers. Differential staining of neurons, glia and oligodendrocytes with time by dual staining with (D) MAP-2 (blue) and Tau (green) and lower images at 2 weeks are of the same field showing MAP-2 being confined to cell bodies and Tau to axons, (E) Pan-NF (blue) and GFAP (green) and (F) oligodendrocyte (blue) and CD45 (green). In (D-F) nuclei were stained with propidium iodide (PI) (red)

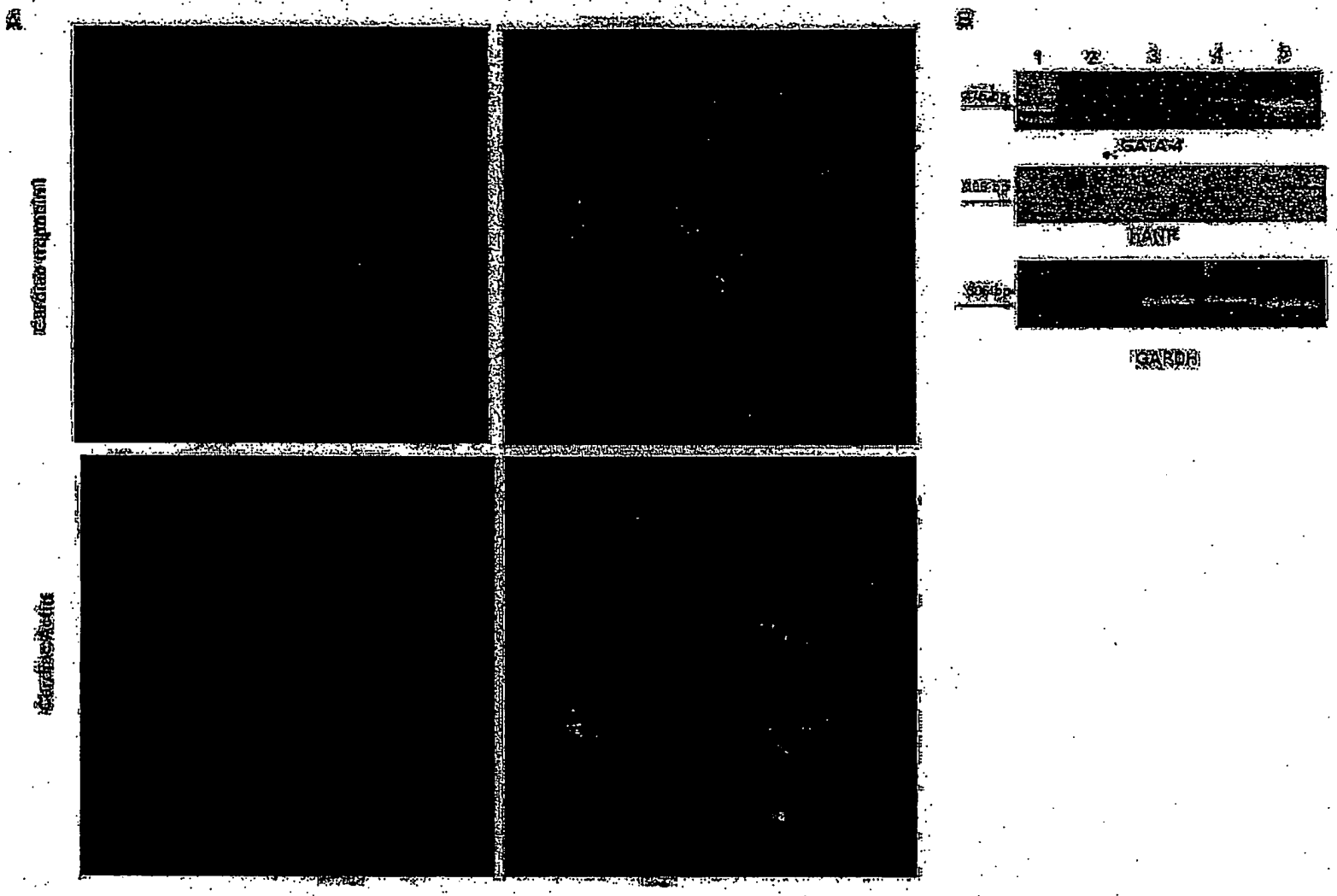

Figure 6. Analysis of cardiac differentiation. (A) phenotypic and (B) time course analysis of genetic changes in MNC cultured in CCC. (A) Differential expression of either cardiac Troponin I or cardiac Actin (both blue) without CD45 (green) after 1 week in culture. Nuclei were stained (red) with PI. (B) RT-PCR analysis of cardiac transcription factors, GATA-4 and hANP and positive control GAPDH-specific transcript expression. Lane orders for each gene transcript: lane 1, 100 bp ladder; lane 2, gene transcript-specific negative control; lane 3, MNC alone; lane 4, 24 h MNC/CCC; lane 5, 1 week MNC/CCC

Discussion

Stem cell plasticity is a newly discovered phenomenon. Recently, cells with multi-developmental potentials have been identified in the bone marrow following *ex vivo* expansion[30]. In contrast, herein, four different types of pluripotent progenitor/stem cells were produced from MNC obtained from unmobilised peripheral adult human blood. In response to the addition of CR3/43 mAb to a variety of well-established culture conditions, HCC, NCC or CCC give rise to haematopoietic, undifferentiated and, subsequently, neuronal or cardiomyogenic progenitor cells. This occurs despite the fact that the source material can be derived from a single blood donor.

Human MNC cultured under HCC are capable of engrafting and differentiating into a variety of lymphohaematopoietic cell lineages in the NOD/SCID mouse model (manuscript in preparation). In addition, such cells when infused into the myocardium of the *Rnu/Rnu* nude rat[49] engrafted and differentiated into fully mature human cardiomyocytes 1 week later (unpublished data), thus further confirming the phenomenon of somatic cell plasticity[2-5,50]. Moreover, a significant proportion of MNC cultured in ES medium supplemented with CR3/43 mAb were converted into cells able to transcribe embryonic stem cells antigen, OCT-4; a transcription factor that is normally restricted in its expression to pluripotent cells[51]. These latter undifferentiated cells can be purified and are able to form EB-like structures in 'hanging drop' cell cultures following proliferation. Continued culturing of MNC in NCC gives rise to fully mature neurons, glia and oligodendrocytes upon extinguishing of nestin and OCT-4, that become capable of secreting a variety of neurotransmitters (unpublished data) – one of the *bona fide* characteristics of neurons. The *in vivo* functional utility of such cells in the reversal of Parkinson's disease in an animal model warrants investigation. Furthermore, the cardiomyogenic progenitor cells produced in response to culturing of MNC in CCC mechanically resemble beating embryoid bodies which appear to give rise to novel cells that are capable of synchronous beating rhythms in 'hanging drop' cell cultures (see supplementary data – Real-time movie B[48]). As mentioned, these cells were able to engraft and differentiate *in vivo* into mature cardiomyocytes. The fact that the cells can follow either a neuronal or cardiomyogenic fate by either culturing against gravity as 'hanging drops' or upright in a six-well plate, with or without artificially introduced extracellular matrix, is particularly noteworthy. This implicates the physical environment in directing the specialisation fate of a group of cells spatially organised in a certain configuration, and profoundly altering their developmental destiny, a notion that may be of importance in engineering and sculpturing human tissue.

The data presented in this report shows for the first time that the phenomenon of somatic cell plasticity is not exclusive to existing stem cells *in vivo* or to embryonic stem cells newly formed via nuclear transfer. Optionally, differentiated cells can also exhibit pluripotency *in vitro*, a notion that may redefine what is a differentiated or a stem cell state. Furthermore, as documented herein, CR3/43 mAb appears to facilitate de-differentiation in MNC[52], while the immediate physical and biochemical surroundings of the cells cause their transdifferentiation or traversal of the differentiation barrier into new and multiple specialisation fates. In this manner, examples whereby signalling through MHC class II has influenced the cell physiology and behaviour include aggregation[53], activation[54,55], proliferation[56-59], anergy[53,60,61] and apoptosis[62,63]. As to why MHC class II cross-linking profoundly alters cell specialisation fate, may be reflected by the differential association of protein kinase C isoforms with HLA-DRβ chains[64]. For example, alternate protein kinase C isoforms have been implicated in controlling differentiation of murine F9 embryonal carcinoma cells: one isoform promotes differentiation into parietal endoderm, whereas another stimulates the retrodifferentiation of such endodermal cells back into multipotent progenitors[65].

The induction of stem cell-like plasticity in a heterogeneous population of leukocytes, predominantly comprising mature specialised cells, may have proceeded by a process of retrodifferentiation[20-25]. This mechanism behind the reprogramming of differentiation in a population of adult cells may be contentious but, nonetheless, may address the phenomena of somatic or stem cell plasticity in haematopoietic stem cells, including the recently described multipotent adult progenitor cells[30]. Irrespective of their origins, the ultimate functional utility of all stem cell types, whatever their source, remains to be determined in diseases where normal physiological functions of a variety of degenerate tissues need to be restored, either in autologous or allogeneic settings.

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